# Nitrate: Nutrient and Signal for Plant Growth

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#### INTRODUCTION

The mineral nutrient needed in greatest abundance by plants is nitrogen. Plants, however, must compete for nitrogen in the soil with abiotic and biotic processes such as erosion, leaching, and microbial consumption. Soil nitrogen is also lost when crops are harvested and plant material is removed from the soil. To be competitive, plants have evolved several mechanisms to acquire nitrogen at low concentrations and to use a variety of forms of nitrogen. Plants can assimilate inorganic forms, such as nitrate and ammonia, and organic forms, such as urea. Some plants, including legumes, can fix dinitrogen gas in association with symbiotic bacteria (see Mylona et al., 1995, this issue). This review focuses on the assimilation of nitrate, a transient yet critical form for plants.

Two key questions arise when considering nitrate assimilation. First, how do plants acquire optimal quantities of nitrate when the soil nitrate concentration can vary from 10  $\mu$ M to 100 mM? Second, how is nitrate assimilation integrated into the overall metabolic program of plants so that optimal amounts of energy and carbon are provided? What is known about nitrate assimilation at the cellular level is summarized in Figure 1. Once taken up, nitrate is either stored in the vacuole or reduced to nitrite by nitrate reductase (NR). Nitrite enters the chloroplast (or plastid in the root) and is then reduced to ammonia by nitrite reductase (NiR). Ammonia can then be fixed into carbon by the action of glutamine synthetase (see Lam et al., 1995, this issue). Reducing energy is provided in the form of NAD(P)H for NR and reduced ferredoxin for NiR. Glutamate provides the immediate carbon skeleton. Because nitrate assimilation produces several hydroxide ions, organic acids are also required for pH homeostasis. Coordinating these steps is a regulatory network that is responsive to both internal and external signals, some of which are diagrammed in Figure 1. Ultimately, it is the understanding of this regulatory network that will answer the aforementioned key questions.

Genetic analysis of nitrate assimilation has been possible because mutants blocked in the pathway can be rescued by providing ammonia as a source of nitrogen. Such mutants are obtained either by screening directly with an in vitro assay for plants defective in nitrate reduction or by selecting plants that are resistant to chlorate, the chlorine analog of nitrate. When chlorate is taken up and reduced by NR, toxic chlorite is produced. In higher plants, chlorate-resistant mutants are impaired in nitrate/chlorate reduction because they have either a defective

nitrate reductase structural gene (*NIA*) or a defective molybdenum cofactor gene (*CNX*). The one exception is the *chl1* mutant of Arabidopsis, which is defective in nitrate/chlorate uptake. Only in fungi and algae have regulatory mutants been described.

There are many excellent recent reviews of the nitrate assimilation field (Pelsy and Caboche, 1992; Warner and Kleinhofs, 1992; Cove, 1993; Crawford and Arst, 1993; Marzluf, 1993; Crawford, 1994; Hoff et al., 1994; Huppe and Turpin, 1994). This review provides an update on recent molecular advances that have uncovered genes and mechanisms responsible for nitrate uptake, reduction, and regulation.

## **NITRATE UPTAKE**

The nitrate uptake system in plants must be versatile and robust because plants have to transport sufficient nitrate to satisfy total demand for nitrogen in the face of external nitrate concentrations that can vary by five orders of magnitude. To function efficiently in the face of such environmental variation, plants have evolved a transport system that is active, regulated, and multiphasic. The energy that drives nitrate uptake comes from the proton gradient maintained across the plasma membrane by the H+-ATPase (Figure 1). The individual components of the nitrate uptake system are just now being discovered, and they are revealing interesting parallels with uptake systems from algae, fungi, and mammals.

The initial uptake of nitrate occurs across the plasma membrane of epidermal and cortical cells of the root (Larsson and Ingemarsson, 1989). Subsequent transport can occur across the tonoplast membrane (Granstedt and Huffaker, 1982; Blumwald and Poole, 1985; Miller and Smith, 1992) and the plasma membrane of cells in the vascular system and leaf (Jackson et al., 1986). This discussion is restricted to the initial uptake process, that is, transport of nitrate across the plasma membrane of root cells.

The transport of anionic nitrate into a root cell must occur up an electrical gradient with potentials that are typically between -100 and -250 mV (negatively charged inside the cell; Figure 1). Given an external nitrate concentration of 2 mM and an electrical potential of -110 mV, one would expect that the

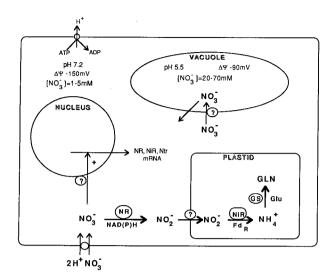


Figure 1. Schematic Diagram of the Nitrate Assimilation Pathway.

The H<sup>+</sup>-ATPase in the plasma membrane pumps protons out of the cell, producing pH and electrical ( $\Delta\Psi$ ) gradients. The nitrate transporters (Ntr) cotransport two or more protons per nitrate into the cell. Nitrate can be transported across the tonoplast and stored in the vacuole. Nitrate in the cytosol is reduced to nitrite, which enters the plastid and is reduced to ammonia. Ammonia is fixed into glutamate (Glu) to produce glutamine (GLN) by the action of glutamine synthetase (GS). Nitrate also acts as a signal to increase the expression of nitrate reductase (NR), nitrite reductase (NR), and Ntr genes.

cytoplasmic concentration of nitrate would be 0.028 mM, based on the Nernst equation. In barley root cells, however, cytoplasmic nitrate concentrations have been recorded between 1 and 5 mM, that is, 35 to 175 times the predicted level (Zhen et al., 1991; King et al., 1992). This indicates that energy is required to drive the transport of nitrate into the cell.

Current evidence strongly indicates that nitrate uptake in plants is driven by electrogenic proton cotransport. During nitrate uptake, the plasma membrane undergoes a rapid and transient depolarization, becoming up to 60 mV more positive inside the cell within 1 to 2 min (Ullrich and Novacky, 1981; McClure et al., 1990a, 1990b; Ullrich and Novacky, 1990; Glass et al., 1992; Tsay et al., 1993b). Application of inhibitors of the plasma membrane H+-ATPase and alkalization of the external medium both inhibit nitrate uptake (McClure et al., 1990a, 1990b). Conversely, acidifying the external medium drives the uptake of chlorate, an analog of nitrate, into plasma membrane vesicles preloaded with chlorate (Ruiz-Cristin and Briskin, 1991).

If only one proton were cotransported with each nitrate anion, no depolarization would be observed. Thus, nitrate uptake involves two or more protons cotransported with every nitrate ion. The depolarization is transient, with the membrane potential beginning to recover within 1 to 2 min after exposure to nitrate. The mechanism for this recovery is not known, but it is likely to result from stimulation of the H+-ATPase. Similar

depolarizations and proton cotransport mechanisms have been observed for chloride and phosphate uptake (Ullrich-Eberius et al., 1981, 1984; Sakano, 1990; Ullrich and Novacky, 1990; Sakano et al., 1992).

Plants have distinct transport systems with different affinities for nitrate. The high affinity system(s) is responsible for nitrate uptake at low concentrations (below  $\sim\!1$  mM). Typically, these systems have saturable kinetics, with  $K_m$  values below 300  $\mu$ M. The lowest reported  $K_m$  values for nitrate uptake are 7  $\mu$ M in barley (Aslam et al., 1992) and 0.2  $\mu$ M in some marine algae (Eppley et al., 1969). These exceptional systems can efficiently scavenge very low levels of nitrate. For example, whereas optimal ryegrass growth requires at least 1.4 mM nitrate, just 14  $\mu$ M nitrate can support growth of ryegrass at 90% optimal levels (Clement et al., 1978).

The cloning of a high affinity nitrate transporter gene has not been reported for higher plants, but three such genes have been isolated from fungi and algae. The Aspergillus CRNA gene was first identified in a search for chlorate-resistant fungi (Cove, 1979). crnA mutants have a two- to fourfold reduction in high affinity nitrate uptake ( $K_{\rm m}$  of 200  $\mu$ M) in young mycelia but can still grow normally on nitrate as the sole nitrogen source (Brownlee and Arst, 1983). CRNA is part of a gene cluster that includes NIAD and NIIA, which encode NR and NiR, respectively (Tomsett and Cove, 1979; Johnstone et al., 1990). CRNA was cloned and found to encode a membrane protein with 10 to 12 putative membrane-spanning segments, as would be expected for a cotransporter (Unkles et al., 1991). It was concluded that CRNA encodes a high affinity nitrate transporter (Unkles et al., 1991). No data on the localization or enzymatic activity of the CRNA gene product are available to confirm this conclusion. Presumably, there are additional Aspergillus proteins that take up nitrate, but none has been described.

Two related transporter genes have been identified in Chlamydomonas. This alga also has a cluster of genes that includes the NR gene, NIT-1 (Quesada et al., 1993). If three of the five genes in the cluster (NAR-2, NAR-3, and NAR-4) are deleted, nitrate uptake at 50 µM is blocked and cells can no longer grow on nitrate (Quesada et al., 1994). Complementation of the triple mutant requires two of the three genes: NAR-2 and NAR-3 or NAR-2 and NAR-4. Sequence analysis shows that the NAR-3 and NAR-4 proteins are almost 80% identical to each other over the region analyzed (the C-terminal 250 amino acids) and are 27 to 31% identical to the CRNA protein of Aspergillus over this region. Thus, Chlamydomonas appears to have two high affinity nitrate transporter genes that are highly related, are located within the same gene cluster, and share similarity with fungal nitrate transporters. No localization or heterologous expression data for the NAR-3 and NAR-4 genes are available. The function of NAR-2 is not known, but it may play a regulatory role.

That the sequences of these three uptake genes are conserved between fungi and algae suggests that higher plants may also have related genes. Recently, such a gene was cloned from barley (Trueman et al., 1994). The cDNA clone pBCH1 encodes a 55-kD protein with 12 putative membrane-spanning

regions that is 32% identical to CRNA and 50% identical to NAR-3. Transcripts of this gene accumulate in roots and become more abundant upon nitrate treatment. As yet, no functional data have been reported for the pBCH1 protein, but it is likely that a member of this gene family will turn out to encode a high affinity nitrate transporter.

In addition to the high affinity transporters, plants have low affinity systems for uptake of nitrate at high concentrations (above  $\sim$ 1 mM). Like the high affinity systems, the low affinity systems are electrogenic and involve proton cotransport (Glass et al., 1992). The low affinity systems, however, often show linear rather than saturable kinetics, have higher  $K_{\rm m}$  values (often 0.5 mM or above), and can be found in both nitrate-treated and untreated plants (Jackson et al., 1973; Doddema and Telkamp, 1979; Behl et al., 1988; Hole et al., 1990; Siddiqi et al., 1990; Aslam et al., 1992, 1993).

The identification of a low affinity nitrate transporter gene came from studies of the CHL1 gene of Arabidopsis. chl1 mutants are resistant to chlorate and show reduced nitrate uptake when external nitrate concentrations exceed 1 mM, the low affinity range (Braaksma and Feenstra, 1973; Doddema and Telkamp, 1979). A T-DNA insertion mutant was used to clone the CHL1 gene, which turned out to encode a membrane protein with 12 putative membrane-spanning segments (Tsay et al., 1993b). Expression of CHL1 in Xenopus oocytes showed that it encodes an electrogenic nitrate transporter whose activity is enhanced by acidifying the external medium. The  $K_{\rm m}$ for nitrate transport by CHL1 in oocytes is high (~8 mM; Y.-F. Tsay and N.M. Crawford, unpublished data), which is consistent with CHL1 encoding a low affinity transporter. CHL1 mRNA is found predominantly in roots, and its level increases when roots are exposed to nitrate or acidic pH. Analysis of chl1 mutants suggests that CHL1 may be involved in the transport of additional ions (Scholten and Feenstra, 1986). The location of CHL1 in the roots has yet to be determined.

CHL1 is a member of an interesting gene family that includes at least four additional related genes. Some of these genes may turn out to encode nitrate transporters. Surprisingly, one of the members encodes a peptide transporter. An Arabidopsis gene, AtPTR2, that was cloned by complementation of a yeast peptide transport mutant (Steiner et al., 1994), encodes a hydrophobic protein with 12 putative membrane-spanning segments that is 25% identical to CHL1. AtPTR2 is also 45% identical to PTR2, a yeast di- and tripeptide transporter (Perry et al., 1994). Among other things, peptide transporters are important for seed germination because they transport storage protein degradation products via the scutellum to embryos (Payne and Walker-Smith, 1987).

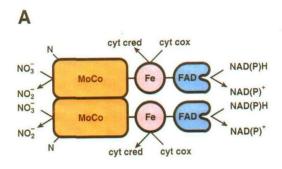
CHL1-related proteins are also involved in animal peptide transport, especially in the intestine. The animal peptide transporter gene *PepT1* was isolated by expression cloning in Xenopus oocytes using a rabbit intestinal cDNA library (Fei et al., 1994). *PepT1* encodes a protein with 12 putative membrane-spanning segments that is ~25% identical to CHL1. Unlike many animal symporters, protons, not sodium, are used to drive the uptake of peptides by PepT1. The *CHL1* gene family

also includes peptide transporters from bacteria and has been named the POT family (proton-dependent oligopeptide transport; Paulsen and Skurray, 1994) and the PTR family (peptide transport; Steiner et al., 1994, 1995). This family is distinct in sequence from the mdr (multidrug resistance) P-glycoprotein gene family, whose members have also been shown to transport peptides and many other solutes, although both families encode proteins with 12 membrane-spanning regions (Hyde et al., 1990; Sharma et al., 1992; Sarkadi et al., 1994). It will be interesting to see whether any of the PTR/POT proteins transport other ions, such as nitrate.

#### **NITRATE REDUCTASE**

After the uptake of nitrate into the cell, the next step in the nitrogen assimilation pathway is reduction of nitrate to nitrite. This step competes with both efflux of nitrate from the cell and transport of nitrate into the vacuole (Figure 1). The enzyme that catalyzes the reduction reaction, NR, is located primarily in the cytosols of root epidermal and cortical cells and shoot mesophyll cells (Rufty et al., 1986; Vaughn and Campbell, 1988; Fedorova et al., 1994). NR transfers two electrons from NAD(P)H to nitrate via three redox centers composed of two prosthetic groups (flavin adenine dinucleotide [FAD] and heme) and a MoCo cofactor, which is a complex of molybdate and pterin. The complexity of NR is reflected in its size: it is a homodimer or homotetramer of 110-kD subunits. Each redox center is associated with a functional domain of the enzyme that has activity independent of the other domains (Figure 2A) (Redinbaugh and Campbell, 1985; Crawford et al., 1988; Campbell and Kinghorn, 1990; Solomonson and Barber, 1990). For example, just two domains of NR, the heme and FAD domains, are needed to catalyze a reaction in which cytochrome c is used instead of nitrate as an alternate electron acceptor. Cytochrome c reductase activity involves transfer of electrons from NAD(P)H to the FAD domain, which reduces the heme domain, which in turn reduces cytochrome c. The MoCo domain need not be intact for this partial reaction to work.

The FAD domain expressed in Escherichia coli has recently been crystallized and found to contain two lobes: an N-terminal lobe containing six antiparallel β-strands that bind directly to the FAD molecule and a C-terminal lobe containing six parallel β-strands that form a cleft with the N-terminal lobe (Figure 2B) (Lu et al., 1994). At the top of the cleft is the FAD, whose isoalloxazine moiety, which accepts the electrons from NAD(P)H, is packed next to the aromatic ring of an invariant tyrosine and is tethered with several hydrogen bonds to the peptide backbone and several side chains. The space underneath the FAD in the cleft is where the NAD(P)H may bind. Comparison of the FAD domain of NR with that of other flavoproteins, such as ferredoxin reductase, indicates that residues in the loop between the β3 and β4 β-strands of the C-terminal lobe may help specify which substrate is bound by the enzyme, NADH or NADPH.



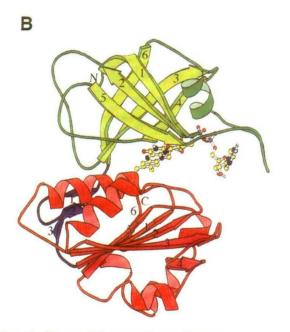


Figure 2. Structural Diagrams of Nitrate Reductase.

(A) A schematic diagram of the NR homodimer with the functional domains indicated. FAD, flavin domain; Fe, heme domain; MoCo, molybdenum cofactor domain; N, N terminus of NR; cyt c, cytochrome c, which can be used as an alternate electron acceptor in vitro; cyt cred and cyt cox, the reduced and oxidized forms of cytochrome c, respectively.

(B) Ribbon model of the structure of the FAD/NADH binding domain of maize NR. This domain contains two lobes, indicated in green and red, that are connected by a short domain shown in purple. The green lobe, which binds the FAD, is N-terminal to the red lobe. The molecule in the cleft is FAD. From Lu et al. (1994) and used with permission of Current Science, Ltd.

The heme domain has also been purified after expression in *E. coli* (Cannons et al., 1993). This isolated domain behaves like the domain in the native enzyme except that it has a more positive "midpoint potential" (+16 mV, compared with -160 mV in the native enzyme). These data indicate that residues outside

the heme domain alter the redox potential of the heme. This potential may be important because transfer of electrons from the heme domain to the MoCo domain may be the rate-limiting step in catalysis (Cannons and Solomonson, 1994). Although the structure of the NR heme domain has not been determined, it bears sequence similarity with mammalian cytochrome  $b_5$  and is therefore likely to have a similar structure (Meyer et al., 1991). A mutation in the *Nicotiana plumbaginifolia* NR gene that completely abolishes activity changes a histidine predicted to be one of the axial ligands for the heme to an asparagine (Meyer et al., 1991).

The FAD and heme domains have been expressed as one recombinant protein without the MoCo domain (Campbell, 1992; Cannons and Solomonson, 1994). This protein retains the same activities (NADH-cytochrome *c* reductase) and enzymatic properties as the corresponding domains in the native enzyme. A crystal structure should be available soon to elucidate how the heme domain interfaces with the FAD domain.

As will be discussed later, NR is tightly regulated at the transcriptional and post-transcriptional levels. Therefore, it is surprising that mutations that reduce NR activity 10-fold have little observable effect on the growth of plants fed only nitrate (Warner et al., 1981; Vaucheret et al., 1990; Wilkinson and Crawford, 1991; Crawford et al., 1992; Dorbe et al., 1992). Why plants produce so much NR protein is a mystery. Overexpression of NR with the cauliflower mosaic virus (CaMV) 35S promoter in transgenic *N. plumbaginifolia* (up to five times the wild-type level) has little effect on growth and metabolism but does reduce leaf nitrate levels (Vincentz and Caboche, 1991; Foyer et al., 1993, 1994; Quillere et al., 1994).

#### **NITRITE REDUCTASE**

The next step in the nitrate assimilation pathway is the reduction of nitrite to ammonia by NiR. NiR is a nuclear-encoded enzyme that is transported into the chloroplast, leaving behind a 30-amino acid transit sequence (Gupta and Beevers, 1987; Back et al., 1988). The holoenzyme is a monomer (60 to 70 kD) with two redox centers: a siroheme-Fe center and an iron-sulfur center. Based on sequence comparisons with other enzymes, the C-terminal half of NiR is thought to contain the redox centers and the N-terminal half is thought to bind the reducing agent ferredoxin (Campbell and Kinghorn, 1990). Ferredoxin reduced by the chloroplast noncyclic electron transport system provides the electrons for reducing nitrite. NiR is also active in roots and is found primarily in proplastids (Suzuki et al., 1985; Bowsher et al., 1989). A ferredoxin-like protein reduced by NADPH from the oxidative pentose phosphate pathway is thought to be the source of reductant.

It has been difficult isolating an NiR mutant in plants because inhibiting nitrite reduction would lead to the accumulation of toxic nitrite (Duncanson et al., 1993). A reduction of NiR levels has been achieved in tobacco by expressing NiR antisense RNA (Vaucheret et al., 1992a). As expected, an antisense transformant with no detectable NiR activity displayed impaired development and chlorotic leaves when fed nitrate and had to be maintained on ammonia as the sole nitrogen source. Interestingly, this transformant had higher and still inducible levels of NR activity and mRNA, perhaps because the levels of a nitrogen metabolite (such as glutamine) that inhibits NR expression are reduced in the transgenic plant.

### **NITRATE AS SIGNAL**

One of the especially interesting features of nitrate assimilation is its regulation. Nitrate serves as the primary signal for regulating nitrate assimilation, but light, cytokinin,  $CO_2$  levels, circadian rhythms, carbon, and nitrogen metabolites such as sucrose and glutamine all play regulatory roles as well. A complex regulatory network involving both transcriptional and post-transcriptional mechanisms serves to integrate nitrate assimilation, which consumes energy and carbon skeletons, with photosynthesis and carbon metabolism. The elucidation of the plant regulatory machinery has been guided by the extensive work with fungal systems, primarily Neurospora and Aspergillus.

One of the universal responses in fungi and plants to nitrate exposure is the induction of gene expression (for review, see Crawford and Arst, 1993; Marzluf, 1993; Hoff et al., 1994). Responding genes encode nitrate transporters, NR, NiR, glutamine synthetase, and ferredoxin-dependent glutamate synthase (Warner and Kleinhofs, 1992; Crawford and Arst, 1993; Redinbaugh and Campbell, 1993; Hoff et al., 1994). In plants, the induction occurs within minutes and in response to very low concentrations of nitrate (down to 10  $\mu$ M). In fungi and algae but not higher plants, ammonia blocks the induction due to catabolite repression.

The study of nitrate regulation in fungi and algae has led to the identification of regulatory genes that are responsible for both nitrate induction and ammonia repression. In fungi, the genes mediating the nitrate induction (NIRA from Neurospora and NIT-4 from Aspergillus) encode positive regulators with zinc finger DNA binding domains similar to GAL4 from yeast (Fu et al., 1989; Burger et al., 1991a, 1991b). The genes mediating ammonia repression (AREA of Aspergillus and NIT-2 of Neurospora) also encode positive regulators with zinc finger DNA binding motifs that are distinct from NIRA and NIT-4 (Fu and Marzluf, 1990a, 1990b; Kudla et al., 1990). In algae, the Chlamydomonas NIT2 regulatory gene, which was isolated by transposon tagging (Schnell and Lefebvre, 1993), encodes a positive regulator whose expression is repressed by ammonia. Thus, catabolite repression of nitrate assimilatory genes in Chlamydomonas may be mediated by the repression of NIT2. No nitrate regulatory genes have been identified in higher plants. However, cis-acting regulatory regions have been detected in the promoters of both the spinach NiR gene and the Arabidopsis NR gene that confer nitrate-inducible expression to reporter genes in transgenic tobacco (Rastogi et al., 1993; Lin et al., 1994).

In addition to nitrate, light is an important regulatory signal for NR and NiR expression. In etiolated plants, a phytochromemediated pathway increases NR and NiR mRNA levels upon transfer to the light (Rajasekhar et al., 1988; Schuster and Mohr, 1990; Seith et al., 1991; Becker et al., 1992; Mohr et al., 1992). In the phytochrome-deficient aurea mutant of tomato, red light induction of NR and NiR genes is reduced (Becker et al., 1992). In cop1 mutants of Arabidopsis, which display a de-etiolated phenotype in the dark, NR mRNA levels are high in the dark, along with those of many other light-regulated genes (X.-W. Deng et al., 1991). In green tissues, NR mRNA levels also increase in the light, but a constitutive low level persists in the dark when nitrate is present. If green plants are placed in a diurnal cycle, the levels of NR and NiR mRNAs oscillate even if the plants are returned to constant light conditions (Galangau et al., 1988; Deng et al., 1990; Bowsher et al., 1991; Cheng et al., 1991; Becker et al., 1992; Pilgrim et al., 1993; Lillo, 1994). Interestingly, if NR is inactivated by inhibitors or by mutation. NR mRNA levels do not oscillate but stay high during the diurnal cycle, even though mRNA levels of other light-regulated genes, such as cab, continue to fluctuate (Deng et al., 1989; Pouteau et al., 1989). Perhaps oscillations in the concentration of a metabolite such as glutamine, which affects NR mRNA levels, cease in NR-deficient plants (M.-D. Deng et al., 1991; Vincentz et al., 1993).

Carbon metabolites signal changes in nitrate assimilation and can supersede the light signal. Carbon metabolites are critical for nitrate reduction because carbon skeletons are required for ammonia fixation and the energy from reduced carbon is needed for nitrate reduction in nongreen tissues. If excised leaves or cells in culture are treated with sucrose in the dark, NR activity and mRNA increase (Aslam and Huffaker, 1984; Cheng et al., 1992; Crawford et al., 1992; Vaucheret et al., 1992b; Vincentz et al., 1993; Lillo, 1994). In addition, NR promoter elements confer on reporter genes sucrose induction in the dark (Cheng et al., 1992; Vincentz et al., 1993). At present, it is not known how sugars induce NR gene expression nor which metabolite is the proximal inducer. Interestingly, NiR mRNA levels do not respond to sugar (Vincentz et al., 1993). Glutamine has a repressive effect on NR mRNA levels (for review, see Hoff et al., 1994).

The regulation discussed previously occurs at the transcriptional level. However, post-transcriptional regulation also occurs, as indicated by the fact that the level of NR mRNA can be uncoupled from the level of NR protein. If tomato plants are starved for nitrogen, for example, the level of NR protein drops to 20% of the induced level within 24 hr, whereas NR mRNA levels remain high for 12 days (Galangau et al., 1988). Similarly, if the NR coding region is constitutively expressed with a CaMV 35S promoter in tobacco, NR protein levels drop four- to fivefold after 72 hr of dark treatment, even though NR mRNA levels remain high (Vincentz and Caboche, 1991). If a mutant form of NR missing 56 amino acids in an acidic region preceding the MoCo domain is constitutively expressed

in the same manner, the levels of the mutant NR do not drop significantly after 72 hr of darkness (Nussaume et al., 1995). These results imply that the structural integrity of the enzyme is needed for post-transcriptional regulation by light and that a sequence in the N-terminal region of the enzyme may play an important role in this regulation.

NR is also regulated by another post-transcriptional mechanism that is both rapid and reversible: phosphorylation. NR activity is rapidly reduced 3- to 10-fold when plants are transferred to the dark or to a low CO2 environment (Kaiser and Brendle-Behnisch, 1991; Huber et al., 1992; Riens and Heldt, 1992). When the light is turned on or normal CO<sub>2</sub> levels are restored, NR activity is restored. Inactivation of NR is mediated by phosphorylation (Kaiser and Spill, 1991; J.L. Huber et al., 1992, 1994; S.C. Huber et al., 1994; MacKintosh, 1992; Kaiser and Huber, 1994b). Spinach, Arabidopsis, and maize NRs have been shown to be phosphorylated on serines, producing multiple phosphorylated peptides when digested with trypsin or cyanogen bromide (J.L. Huber et al., 1992, 1994; LaBrie and Crawford, 1994). Phosphorylation of two (in spinach) or three (in maize) of these peptides increases when NR is inactivated by the dark treatment (J.L. Huber et al., 1992, 1994). Reactivation of NR correlates with dephosphorylation of these peptides, which can be blocked with phosphatase inhibitors (Huber et al., 1992; MacKintosh, 1992; Kaiser and Huber, 1994a). Thus, phosphorylation plays an important role in modulating NR activity rapidly and reversibly in response to carbon and light signals. This regulation of NR may be critical to prevent the accumulation of toxic nitrite when levels of reduced ferredoxin become low enough, especially in the dark, to restrict nitrite reduction in the plastid.

Preliminary evidence suggests that phosphorylation involves the MoCo domain of NR. Partial activity assays using methyl viologen instead of NADH as the electron donor show that electron flow from the heme via the MoCo domain to nitrate is inhibited in phosphorylated NR (Huber et al., 1992). In addition, a mutation in the MoCo domain of NR can drastically impair NR phosphorylation (LaBrie and Crawford, 1994). This mutation converts a glycine to an aspartic acid and reduces phosphate levels to 10 to 20% of the wild-type level. Because the mutant NR is inactive, it has not been possible to determine how reduced phosphorylation affects NR regulation. However, because a single alteration in the MoCo domain can disrupt phosphorylation, possibly at multiple sites, the MoCo domain is likely to be a target of the phosphorylation. More experiments are needed to pinpoint the key regulatory serines that are phosphorylated.

Inactivation of NR involves not only a kinase but also an inhibitory protein. When inactivated NR from dark-treated spinach is partially purified, NR activity is restored even though NR is still phosphorylated (MacKintosh et al., 1995). Other fractions from the purification inactivate NR when mixed with the partially purified, active NR fraction. The inhibitory fractions contain a protein called NIP that stoichiometrically inactivates NR when it is phosphorylated but has no effect on dephosphorylated NR. In another study, two protein fractions,

containing 100-kD and 67-kD proteins, were found to be necessary for inactivating spinach NR in vitro in the presence of ATP (Spill and Kaiser, 1994). The 67-kD fraction appears to contain the NR kinase, and the 100-kD fraction may contain NIP.

#### **CONCLUSIONS AND FUTURE PROSPECTS**

At least one player of each step of the nitrate assimilation pathway has been identified and cloned. In the next year or two, genes that encode plant high affinity nitrate transporters and the plant MoCo enzymes will be isolated and characterized. Already, two MoCo biosynthetic genes from Arabidopsis have been cloned by complementing E. coli MoCo mutants (Hoff et al., 1995). Expression systems will be perfected to produce quantities of NR sufficient for crystallization and structure determination. These systems will also allow the production of mutant forms of NR that will help us to better understand how the enzyme functions and is regulated. Progress has been made in our understanding of the regulatory mechanisms in this pathway, especially the role of phosphorylation in controlling NR activity, but regulatory mutants, transcription factors, nitrate receptors, and signal transduction mechanisms still need to be elucidated.

As we learn more about the basic biology and chemistry of nitrate assimilation, this system will continue to provide important tools for both basic and applied research. For example, the NR gene has been used as a selectable marker for fungal and algal transformation (Kindle et al., 1989; Unkles et al., 1989). In higher plants, the NR coding region has been fused to the CaMV 35S promoter (Nussaume et al., 1991) and to the promoter of the light-harvesting chlorophyll a/b protein (Heimer et al., 1995) for use in counterselections and mutant isolations. Several nitrate assimilatory genes, including NIA and CHL1, have been used successfully as transposon traps (Grandbastien et al., 1989; Tsay et al., 1993a). For the future, the NR enzyme holds promise for use in nitrate determination and elimination devices for dealing with nitrate pollution in drinking water. For example, an experimental bioreactor with immobilized NR has been developed that reduces nitrate to dinitrogen gas using electrical currents (Mellor et al., 1992). The hope that major crop plants will be engineered to require less nitrogen fertilizer for optimal yield is still alive because we now have some of the critical genes that control nitrate assimilation and can alter their expression in vivo.

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#### REFERENCES

- Asiam, M., and Huffaker, R.C. (1984). Dependency of nitrate reduction on soluble carbohydrates in primary leaves of barley under aerobic conditions. Plant Physiol. 75, 623–628.
- Aslam, M., Travis, R.L., and Huffaker, R.C. (1992). Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley seedlings. Plant Physiol. 99, 1124–1133
- Aslam, M., Travis, R., and Huffaker, R.C. (1993). Comparative induction of nitrate and nitrite uptake and reduction systems by ambient nitrate and nitrite in intact roots of barley seedlings. Plant Physiol. 102, 811–819
- Back, E., Burkhart, W., Moyer, M., Privalle, L., and Rothstein, S. (1988). Isolation of cDNA clones coding for spinach nitrite reductase: Complete sequence and nitrate induction. Mol. Gen. Genet. 212, 20–26.
- Becker, T.W., Foyer, C., and Caboche, M. (1992). Light-regulated expression of the nitrate reductase and nitrite reductase genes in tomato and in the phytochrome-deficient aurea mutant of tomato. Planta 188, 39–47.
- Behl, R., Tischner, R., and Raschke, K. (1988). Induction of a high capacity nitrate uptake mechanism in barley roots prompted by nitrate uptake through a constitutive low capacity system. Planta 176, 235–240.
- Blumwald, E., and Poole, R.J. (1985). Nitrate storage and retrieval in *Beta vulgaris*: Effects of nitrate and chloride on proton gradients in tonoplast vesicles. Proc. Natl. Acad. Sci. USA 82, 3683–3687.
- Bowsher, C.G., Hucklesby, D.P., and Emes, M.J. (1989). Nitrite reduction and carbohydrate metabolism in plastids purified from roots of *Pisum sativum*. Planta 177, 359–366.
- Bowsher, C.G., Long, D.M., Oaks, A., and Rothstein, S.J. (1991). Effect of light/dark cycles on expression of nitrate assimilatory genes in maize shoots and roots. Plant Physiol. 95, 281–285.
- Braaksma, F.J., and Feenstra, W.J. (1973). Isolation and characterization of chlorate resistant mutants of *Arabidopsis thaliana*. Mutat. Res. 19, 175–185.
- Brownlee, A.G., and Arst, H.N., Jr. (1983). Nitrate uptake in *Aspergillus nidulans* and involvement of the third gene of the nitrate assimilation gene cluster. J. Bacteriol. **155**, 1138–1146.
- Burger, G., Tilburn, J., and Scazzocchio, C. (1991a). Molecular cloning and functional characterization of the pathway-specific regulatory gene nirA, which controls nitrate assimilation in Aspergillus nidulans. Mol. Cell. Biol. 11, 795~802.
- Burger, G., Strauss, J., Scazzocchio, C., and Lang, B.F. (1991b). nirA, the pathway-specific regulatory gene of nitrate assimilation in Aspergillus nidulans, encodes a putative GAL4-type zinc finger protein and contains four introns in highly conserved regions. Mol. Cell. Biol. 11, 5746–5755.

- Campbell, W.H. (1992). Expression in Escherichia coli of cytochrome c reductase activity from a maize NADH:nitrate reductase complementary DNA. Plant Physiol. 99, 693–699.
- Campbell, W.H., and Kinghorn, J.R. (1990). Functional domains of assimilatory nitrate reductases and nitrite reductases. Trends Biochem. Sci. 15, 315–319.
- Cannons, A.C., and Solomonson, L.P. (1994). Heterologous expression of functional domains of assimilatory nitrate reductase. J. Protein Chem. 13, 445–447.
- Cannons, A.C., Barber, M.J., and Solomonson, L.P. (1993). Expression and characterization of the heme-binding domain of *Chlorella* nitrate reductase. J. Biol. Chem. 268, 3268–3271.
- Cheng, C.-L., Acedo, G.N., Dewdney, J., Goodman, H.M., and Conkling, M.A. (1991). Differential expression of the two *Arabidopsis* nitrate reductase genes. Plant Physiol. **96**, 275–279.
- Cheng, C.-L., Acedo, G.N., Cristinsin, M., and Conkling, M.A. (1992).
  Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. Proc. Natl. Acad. Sci. USA 89, 1861–1864.
- Clement, C.R., Hopper, M.J., Jones, L.H., and Leafe, E.L. (1978).
  The uptake of nitrate by *Lolium perenne* from flowing nutrient solution. J. Exp. Bot. 29, 1173–1178.
- Cove, D.J. (1979). Genetic studies of nitrate assimilation in Aspergillus nidulans. Biol. Rev. **54**, 291–327.
- Cove, D.J. (1993). Mutant analysis, a key tool for the study of metabolism and development. Plant J. 3, 303–308.
- Crawford, N.M. (1994). Metabolic and genetic control of nitrate, phosphate, and iron assimilation in plants. In Arabidopsis, E.M. Meyerowitz and C.R. Somerville, eds (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 1119–1146.
- Crawford, N.M., and Arst, H.N.J. (1993). The molecular genetics of nitrate assimilation in fungi and plants. Annu. Rev. Genet. 27, 115–146.
- Crawford, N.M., Smith, M., Bellissimo, D., and Davis, R.W. (1988).
  Sequence and nitrate regulation of the Arabidopsis thaliana mRNA encoding nitrate reductase, a metalloflavoprotein with three functional domains. Proc. Natl. Acad. Sci. USA 85, 5006–5010.
- Crawford, N.M., Wilkinson, J.Q., and LaBrie, S.T. (1992). Metabolic control of nitrate reduction in *Arabidopsis thaliana*. Aust. J. Plant Physiol. 19, 377–385.
- Deng, M., Moureaux, T., and Caboche, M. (1989). Tungstate, a molybdate analog inactivating nitrate reductase, deregulates the expression of the nitrate reductase structural gene. Plant Physiol. 91, 304–309.
- Deng, M.-D., Moureaux, T., Leydecker, M.-T., and Caboche, M. (1990).
  Nitrate-reductase expression is under the control of a circadian rhythm and is light inducible in *Nicotiana tabacum* leaves. Planta 180, 281–285.
- Deng, M.-D., Moureaux, T., Cherel, I., Boutin, J.P., and Caboche, M. (1991). Effects of nitrogen metabolites on the regulation and circadian expression of tobacco nitrate reductase. Plant Physiol. Biochem. 29, 239–247.
- Deng, X.-W., Caspar, T., and Quail, P.H. (1991). cop1: A regulatory locus involved in light-controlled development and gene expression in Arabidopsis. Genes Dev. 5, 1172–1182.
- Doddema, H., and Telkamp, G.P. (1979). Uptake of nitrate by mutants of *Arabidopsis thaliana*, disturbed in uptake or reduction of nitrate. II. Kinetics. Physiol. Plant. 45, 332–338.
- Dorbe, M.-F., Caboche, M., and Daniel-Vedele, F. (1992). The tomato nia gene complements a Nicotiana plumbaginifolia nitrate reductase-

- deficient mutant and is properly regulated. Plant Mol. Biol. 18, 363-375.
- Duncanson, E., Gilkes, A.F., Kirk, D.W., Sherman, A., and Wray, J.L. (1993). nir1, a conditional-lethal mutation in barley causing a defect in nitrite reduction. Mol. Gen. Genet. 236, 275–282.
- Eppley, R.W., Rogers, J.N., and McCarthy, J.J. (1969). Half-saturation constants for uptake of nitrate and ammonium by marine phytoplankton. Limnol. Oceanogr. 14, 912–920.
- Fedorova, E., Greenwood, J.S., and Oaks, A. (1994). In-situ localization of nitrate reductase in maize roots. Planta 194, 279–286.
- Fei, Y.-J., Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F.H., Romero, M.F., Singh, S.K., Boron, W.F., and Hediger, M.A. (1994). Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature 368, 563-566.
- Foyer, C.H., Lefebvre, C., Provot, M., Vincentz, M., and Vaucheret, H. (1993). Modulation of nitrogen and carbon metabolism in transformed *Nicotiana plumbaginifolia* mutant E23 lines expressing either increased or decreased nitrate reductase activity. Aspects Appl. Biol. 34, 137–145.
- Foyer, C.H., Lescure, J.-C., Lefebvre, C., Morot-Gaudry, J.-F., Vincentz, M., and Vaucheret, H. (1994). Adaptations of photosynthetic electron transport, carbon assimilation and carbon partitioning in transgenic *Nicotiana plumbaginifolia* plants to changes in nitrate reductase activity. Plant Physiol. 104, 171–178.
- Fu, Y.-H., and Marzluf, G.A. (1990a). nit-2, the major nitrogen regulatory gene of Neurospora crassa, encodes a protein with a putative zinc finger DNA-binding domain. Mol. Cell. Biol. 10, 1056–1065.
- Fu, Y.-H., and Marzluf, G.A. (1990b). nit-2, the major positive acting nitrogen regulatory gene of Neurospora crassa, encodes a sequencespecific DNA-binding protein. Proc. Natl. Acad. Sci. USA 87, 5331–5335.
- Fu, Y.-H., Kneesi, J.Y., and Marzluf, G.A. (1989). Isolation of nit-4, the minor nitrogen regulatory gene which mediates nitrate induction in Neurospora crassa. J. Bacteriol. 171, 4067–4070.
- Galangau, F., Daniel-Vedele, F., Moureaux, T., Dorbe, M.-F., Leydecker, M.-T., and Caboche, M. (1988). Expression of leaf nitrate reductase genes from tomato and tobacco in relation to light-dark regimes and nitrate supply. Plant Physiol. 88, 383–388.
- Glass, A.D., Shaff, J.E., and Kochlan, L.V. (1992). Studies of the uptake of nitrate in barley. IV. Electrophysiology. Plant Physiol. 99, 456–463.
- Grandbastien, M.A., Spielmann, A., and Caboche, M. (1989). Tnt1, a mobile retroviral-like transposable element of tobacco isolated by plant cell genetics. Nature 337, 376–380.
- Granstedt, R.C., and Huffaker, R.C. (1982). Identification of the leaf vacuole as a major nitrate storage pool. Plant Physiol. 70, 410–413.
- Gupta, S.C., and Beevers, L. (1987). Regulation of nitrite reductase, cell-free translation and processing. Plant Physiol. 83, 750–754.
- Heimer, Y.M., Brusslan, J.A., Kenigsbuch, D., and Tobin, E.M. (1995).
  A chimeric Lhcb::Nia gene: An inducible counter selection system for mutants in the phytochrome signal transduction pathway. Plant Mol. Biol. 27, 129–136.
- Hoff, T., Truon, H.-M., and Caboche, M. (1994). The use of mutants and transgenic plants to study nitrate assimilation. Plant Cell Environ. 17, 489–506.
- Hoff, T., Schnorr, K.M., Meyer, C., and Caboche, M. (1995). Isolation of two Arabidopsis cDNAs involved in early steps in molybdenum

- cofactor biosynthesis by functional complementation of *Escherichla coli* mutants. J. Biol. Chem. **270**, 6100–6107.
- Hole, D., Emran, A.M., Fares, Y., and Drew, M.C. (1990). Induction of nitrate transport in maize roots, and kinetics of influx, measured with nitrogen-13. Plant Physiol. 93, 642–647.
- Huber, J.L., Huber, S.C., Campbell, W.H., and Redinbaugh, M.G. (1992). Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. Arch. Biochem. Biophys. 296, 58–65.
- Huber, J.L., Redinbaugh, M.G., Huber, S.C., and Campbell, W.H. (1994). Regulation of maize leaf nitrate reductase activity involves both gene expression and protein phosphorylation. Plant Physiol. 106. 1667–1674.
- Huber, S.C., Huber, J.L., and McMichael, R.W. (1994). Control of plant enzyme activity by reversible protein phosphorylation. Int. Rev. Cytol. 149. 47–98.
- Huppe, H.C., and Turpin, D.H. (1994). Integration of carbon and nitrogen metabolism in plant and algal cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 577–607.
- Hyde, S.C., Emsley, P., Harshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E., and Higgins, C.F. (1990). Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 346, 362–365.
- Jackson, W.A., Flesher, D., and Hageman, R.H. (1973). Nitrate uptake by dark-grown maize seedlings. Plant Physiol. 51, 120–127.
- Jackson, W.A., Pan, W.A., Moll, R.H., and Kamprath, E.J. (1986).
  Uptake, translocation and reduction of nitrate. In Biochemical Basis of Plant Breeding, C. Neyra, ed (Boca Raton, FL: CRC Press), pp. 73–108.
- Johnstone, I.L., McCabe, P.C., Greaves, P., Gurr, S.J., Cole, G.E., Brow, M.A.D., Unkles, S.E., Clutterbuck, A.J., Kinghorn, J.R., and Innis, M.A. (1990). Isolation and characterization of the crnAniiA-niaD gene cluster for nitrate assimilation in Aspergillus nidulans. Gene 90, 181–192.
- Kaiser, W.M., and Brendle-Behnisch, E. (1991). Rapid modulation of spinach leaf nitrate reductase activity by photosynthesis. I. Modulation in vivo by CO<sub>2</sub> availability. Plant Physiol. **96**, 363–367.
- Kaiser, W.M., and Huber, S. (1994a). Modulation of nitrate reductase in vivo and in vitro: Effects of phosphoprotein phosphatase inhibitors, free Mo<sup>2+</sup> and 5'-AMP, Planta 193, 358–364.
- Kaiser, W.M., and Huber, S.C. (1994b). Posttranslational regulation of nitrate reductase in higher plants. Plant Physiol. 106, 817–821.
- Kaiser, W.M., and Spill, D. (1991). Rapid modulation of spinach leaf nitrate reductase by photosynthesis. II. In vitro modulation by ATP and AMP. Plant Physiol. 96, 368–375.
- Kindle, K.L., Schnell, R.A., Fernandez, E., and Lefebvre, P.A. (1989).
  Stable nuclear transformation of Chlamydomonas using the Chlamydomonas gene for nitrate reductase. J. Cell Biol. 109, 2589–2601.
- King, B.J., Siddlqi, M.Y., and Glass, A.D. (1992). Studies of the uptake of nitrate in barley. V. Estimation of root cytoplasmic nitrate concentration using nitrate reductase activity—implications for nitrate influx. Plant Physiol. 99, 1582–1589.
- Kudla, B., Caddick, M.X., Langdon, T., Martinez-Rossi, N. M., Bennett, C.F., Sibley, S., Davies, R.W., and Arst, H.N., Jr. (1990). The regulatory gene areA mediating nitrogen metabolite repression

- in Aspergillus nidulans: Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. EMBO J. 9, 1355–1364.
- LaBrie, S.T., and Crawford, N.M. (1994). A glycine to aspartic acid change in the MoCo domain of nitrate reductase reduces both activity and phosphorylation levels in *Arabidopsis*. J. Biol. Chem. 269, 14497–14501.
- Lam, H.-M., Coschigano, K., Schultz, C., Melo-Oliveira, R., Tjaden, G., Oliveira, I., Ngai, N., Hsieh, M.-H., and Coruzzi, G. (1995).
  Use of Arabidopsis mutants and genes to study amide amino acid biosynthesis. Plant Cell 7, 887–898.
- Larsson, C.-M., and Ingemarsson, B. (1989). Molecular aspects of nitrate uptake in higher plants. In Molecular and Genetic Aspects of Nitrate Assimilation, J.L. Wray and J.R. Kinghorn, eds (Oxford: Oxford Science Publications), pp. 3–14.
- Lillo, C. (1994). Light regulation of nitrate reductase in green leaves of higher plants. Physiol. Plant. 90, 616–620.
- Lin, Y., Hwang, C.-F., Brown, J.B., and Cheng, C.-L. (1994). 5' proximal regions of *Arabidopsis* nitrate reductase genes direct nitrate-induced transcription in transgenic tobacco. Plant Physiol. 106, 477–484.
- Lu, G., Campbell, W.H., Schneider, G., and Lindqvist, Y. (1994).
  Crystal structure of the FAD-containing fragment of corn nitrate reductase at 2.5 Å resolution: Relationship to other flavoprotein reductases. Structure 2, 809–821.
- MacKintosh, C. (1992). Regulation of spinach leaf nitrate reductase by reversible phosphorylation. Biochem. Biophys. Acta 1137, 121–126.
- MacKintosh, C., Douglas, P., and Lillo, C. (1995). Identification of a protein that inhibits the phosphorylated form of nitrate reductase from spinach leaves. Plant Physiol. 107, 451–457.
- Marzluf, G.A. (1993). Regulation of sulfur and nitrogen metabolism in filamentous fungi. Annu. Rev. Microbiol. 47, 31–55.
- McClure, P.R., Kochian, L.V., Spanswick, R.M., and Shaff, J.E. (1990a). Evidence for cotransport of nitrate and protons in maize roots. I. Effects of nitrate on the membrane potential. Plant Physiol. 93, 281–289.
- McClure, P.R., Kochian, L.V., Spanswick, R.M., and Shaff, J.E. (1990b). Evidence for cotransport of nitrate and protons in maize roots. II. Measurement of NO<sub>3</sub><sup>-</sup> and H<sup>+</sup> fluxes with ion-selective microelectrodes. Plant Physiol. **93**, 290–294.
- Mellor, R.B., Ronnenberg, J., Campbell, W.H., and Diekmann, S. (1992). Reduction of nitrate and nitrite in water by immobilized enzymes. Nature 355, 717–719.
- Meyer, C., Levin, J.M., Roussel, J.M., and Rouze, P. (1991). Mutational and structural analysis of the nitrate reductase heme domain of Nicotiana plumbaginifolia. J. Biol. Chem. 266, 20561–20566.
- Miller, A.J., and Smith, S.J. (1992). The mechanism of nitrate transport across the tonoplast of barley root cells. Planta 187, 554–557.
- Mohr, H., Neineinger, A., and Selth, B. (1992). Control of nitrate reductase and nitrite reductase gene expression by light, nitrate and a plastidic factor. Bot. Acta 105, 81–89.
- Mylona, P., Pawlowski, K., and Bisseling, T. (1995). Symbiotic nitrogen fixation. Plant Cell 7, 869–885.
- Nussaume, L., Vincentz, M., and Caboche, M. (1991). Constitutive nitrate reductase a dominant conditional marker for plant genetics. Plant J. 1, 267–274.

- Nussaume, L., Vincentz, M., Meyer, C., Boutin, J.-P., and Caboche, M. (1995). Post-transcriptional regulation of nitrate reductase by light is abolished by an N-terminal deletion. Plant Cell 7, 611–621.
- Paulsen, I.T., and Skurray, R.A. (1994). The POT family of transport proteins. Trends Biochem. Sci. 19, 404.
- Payne, J.W., and Walker-Smith, D.J. (1987). Isolation and identification of proteins from the peptide transport carrier in the scutellum of germinating barley embryos. Planta 170, 263–271.
- Pelsy, F., and Caboche, M. (1992). Molecular genetics of nitrate reductase in higher plants. Adv. Genet. 30, 1–40.
- Perry, J.R., Basral, M.A., Steiner, H.-Y., Naider, R., and Becker, J.M. (1994). Isolation and characterization of a Saccharomyces cerevisiae peptide transport gene. Mol. Cell. Biol. 14, 104–115.
- Pilgrim, M.L., Caspar, T., Quall, P.H., and McClung, C.R. (1993).
  Circadian and light-regulated expression of nitrate reductase in Arabidopsis. Plant Mol. Biol. 23, 349–364.
- Pouteau, S., Chérel, I., Vaucheret, H., and Caboche, M. (1989). Nitrate reductase mRNA regulation in *Nicotiana plumbaginifolia* nitrate reductase-deficient mutants. Plant Cell 1, 1111–1120.
- Quesada, A., Galvan, A., Schnell, R.A., Lefebvre, P.A., and Fernandez, E. (1993). Five nitrate assimilation related genes are clustered in *Chlamydomonas reinhardtii*. Mol. Gen. Genet. 240, 387–394.
- Quesada, A., Galvan, A., and Fernandez, E. (1994). Identification of nitrate transporter genes in *Chlamydomonas reinhardtii*. Plant J. 5. 407–419.
- Quillere, I., Dufosse, C., Roux, Y., Foyer, C.H., Caboche, M., and Morot-Gaudry, J.-F. (1994). The effects of deregulation of NR gene expression on growth and nitrogen metabolism of *Nicotiana plum-baginifolia* plants. J. Exp. Bot. 45, 1205–1211.
- Rajasekhar, V.K., Gowri, G., and Campbell, W.H. (1988). Phytochrome-mediated light regulation of nitrate reductase expression in squash cotyledons. Plant Physiol. 88, 242–244.
- Rastogi, R., Back, E., Schnelderbauer, A., Bowsher, C., Moffatt, B., and Rothstein, S.J. (1993). A 330-bp region of the spinach nitrite reductase gene promoter directs nitrate-inducible tissue specific expression in transgenic tobacco. Plant J. 4, 317–326.
- Redinbaugh, M.G., and Campbell, W.H. (1985). Quaternary structure and composition of squash NADH:nitrate reductase. J. Biol. Chem. 260, 3380–3385.
- Redinbaugh, M.G., and Campbell, W.H. (1993). Glutamine synthetase and ferredoxin-dependent glutamate synthase expression in the maize (*Zea mays*) root primary response to nitrate. Plant Physiol. 101. 1249–1255.
- Riens, B., and Heldt, H.W. (1992). Decrease of nitrate reductase activity in spinach leaves during a light-dark transition. Plant Physiol. 98, 573–577.
- Rufty, T.W., Thomas, J.E., Remmler, J.L., Campbell, W.H., and Volk, R.J. (1986). Intracellular localization of nitrate reductase in roots. Plant Physiol. **82**, 675–680.
- Ruiz-Cristin, J., and Briskin, D.P. (1991). Characterization of a H<sup>+</sup>/NO<sub>3</sub><sup>-</sup> symport associated with plasma membrane vesicles of maize roots using <sup>36</sup>ClO<sub>3</sub><sup>-</sup> as a radiotracer analog. Arch. Biochem. Biophys. **285**, 74–82.
- Sakano, K. (1990). Proton/phosphate stoichiometry in uptake of inorganic phosphate by cultured cells of *Catharanthus roseus*. Plant Physiol. 93, 479–483.

- Sakano, K., Yazake, Y., and Mimura, T. (1992). Cytoplasmic acidification induced by inorganic phosphate uptake in suspension cultured Catharanthus roseus cells. Plant Physiol. 99, 672–680.
- Sarkadi, B., Muller, M., Homolya, L., Hollo, Z., Seprodi, J., Germann, U.A., Gottesman, M.M., Price, E.M., and Boucher, R.C. (1994). Interaction of bioactive hydrophobic peptides with the human multi-drug transporter. FASEB J. 8, 766–770.
- Schnell, R.A., and Lefebvre, P.A. (1993). Isolation of the Chlamydomonas regulatory gene NIT2 by transposon tagging. Genetics 134, 737–747.
- Scholten, H.J., and Feenstra, W.J. (1986). Uptake of chlorate and other ions in seedlings of the nitrate uptake mutant B1 of Arabidopsis thaliana. Physiol. Plant. 66, 265–269.
- Schuster, C., and Mohr, H. (1990). Appearance of nitrite reductase mRNA in mustard seedling cotyledons is regulated by phytochrome. Planta 181, 327–334.
- Seith, B., Schuster, C., and Mohr, H. (1991). Coaction of light, nitrate and a plastidic factor in controlling nitrite reductase gene expression in spinach. Planta 184, 74–80.
- Sharma, R.C., Inoue, S., Roitelman, J., Schimke, R.T., and Simoni, R.D. (1992). Peptide transport by the multidrug resistance pump. J. Biol. Chem. 267, 5731–5734.
- Siddiqi, M.Y., Glass, A.D., Ruth, T.J., and Rufty, T.W. (1990). Studies of the uptake of nitrate in barley. Plant Physiol. 93, 1426-1432.
- Solomonson, L.P., and Barber, M.J. (1990). Assimilatory nitrate reductase: Functional properties and regulation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 225–253.
- Spill, D., and Kaiser, W.M. (1994). Partial purification of two proteins (100 kDa and 67 kDa) cooperating in the ATP-dependent inactivation of spinach leaf nitrate reductase. Planta 192, 183–188.
- Steiner, H.-Y., Song, W., Zhang, L., Naider, F., Becker, J.M., and Stacey, G. (1994). An Arabidopsis peptide transporter is a member of a new class of membrane transport proteins. Plant Cell 6, 1289–1299.
- Steiner, H.-Y., Naider, F., and Becker, J.M. (1995). The PTR family— A new group of peptide transporters. Mol. Microbiol., in press.
- Suzuki, A., Oads, A., Jacquot, J.P., Vidal, J., and Gadal, P. (1985).
  An electron transport system in maize roots for reactions of glutamate synthase and nitrite reductase. Plant Physiol. 78, 374–378.
- Tomsett, A.B., and Cove, D.J. (1979). Deletion mapping of the niiA niaD gene region of Aspergillus nidulans. Genet. Res. 34, 19–32.
- Trueman, L.J., Theodoulou, R.L., Miller, A.J., and Forde, B.G. (1994).
  Cloning and analysis of a putative high affinity nitrate transporter from barley. Abstracts of the Fourth International Congress of Plant Molecular Biology. (Amsterdam: The International Society for Plant Molecular Biology), abstr. 1007.
- Tsay, Y.-F., Frank, M.J., Page, T., Dean, C., and Crawford, N.M. (1993a). Identification of a mobile endogenous transposon in Arabidopsis thaliana. Science 260, 342–344.
- Tsay, Y.-F., Schroeder, J.I., Feldmann, K.A., and Crawford, N.M. (1993b). A herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter. Cell 72, 705–713.

- Ullrich, C.I., and Novacky, A.J. (1990). Extra- and intracellular pH and membrane potential changes induced by K+, Cl<sup>-</sup>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup> uptake and fusicoccin in root hairs of *Limnobium storonife-rum*. Plant Physiol. **94**, 1561–1567.
- Ullrich, W.R., and Novacky, A. (1981). Nitrate-dependent membrane potential changes and their induction in *Lemna gibba* G1. Plant Sci. Lett. 22, 211–217.
- Ullrich-Eberius, C., Novacky, C., Fischer, E., and Luttge, U. (1981).
  Relationship between energy-dependent phosphate uptake and the electrical membrane potential in *Lemna gibba* G1. Plant Physiol. 67, 797–801.
- Ullrich-Eberius, C., Novacky, C., and van Bell, A.J. (1984). Phosphate uptake in *Lemna gibba* G1: Energetics and kinetics. Planta 161, 43–52.
- Unkles, S.E., Campbell, E.I., Carrez, D., Grleve, C., Contreras, R., Fiers, W., Van den Hondel, C., and Kinghorn, J.R. (1989). Transformation of Aspergillus niger with the homologous nitrate reductase gene. Gene 78, 157–166.
- Unkles, S.E., Hawker, K.L., Grieve, C., Campbell, E.I., Montague, P., and Kinghorn, J.R. (1991). crnA encodes a nitrate transporter in Aspergillus nidulans. Proc. Natl. Acad. Sci. USA 88, 204–208.
- Vaucheret, H., Chabaud, M., Kronenberger, J., and Caboche, M. (1990). Functional complementation of tobacco and Nicotiana plumbaginifolia nitrate reductase deficient mutants by transformation with the wild-type alleles of the tobacco structural genes. Mol. Gen. Genet. 220, 468–474.
- Vaucheret, H., Kronenberger, J., Lepingle, A., Vilaine, F., Boutin, J.-P., and Caboche, M. (1992a). Inhibition of tobacco nitrite reductase activity by expression of antisense RNA. Plant J. 2, 559–569.
- Vaucheret, H., Marion-Poll, A., Meyer, C., Faure, J.-D., Marin, E., and Caboche, M. (1992b). Interest in and limits to the utilization of reporter genes for the analysis of transcriptional regulation of nitrate reductase. Mol. Gen. Genet. 235, 259–268.
- Vaughn, K.C., and Campbell, W.H. (1988). Immunogold localization of nitrate reductase in maize leaves. Plant Physiol. 88, 1354–1357.
- Vincentz, M., and Caboche, M. (1991). Constitutive expression of nitrate reductase allows normal growth and development of *Nicotiana plumbaginifolia* plants. EMBO J. 10, 1027–1035.
- Vincentz, M., Moureaux, T., Leydecker, M.-T., Vaucheret, H., and Caboche, M. (1993). Regulation of nitrate and nitrite reductase expression in Nicotiana plumbaginifolia leaves by nitrogen and carbon metabolites. Plant J. 3, 315–324.
- Warner, R.L., and Kleinhofs, A. (1992). Genetics and molecular biology of nitrate metabolism. Physiol. Plant. 85, 245–252.
- Warner, R.L., Lin, C.J., and Kleinhofs, A. (1981). Nitrate reductasedeficient mutants in barley. Nature 269, 406–407.
- Wilkinson, J.Q., and Crawford, N.M. (1991). Identification of the Arabidopsis CHL3 gene as the nitrate reductase structural gene NIA2. Plant Cell 3, 461–471.
- Zhen, R.G., Koyro, H.-W., Leigh, R.A., Tomos, A.D., and Miller, A.J. (1991). Compartmental nitrate concentration in barley root cells measured with nitrate-selective microelectrodes and by single-cell sap sampling. Planta 185, 356–361.